Chapter 3

Markers for Sperm Freezability and Relevance of Transcriptome Studies in Semen Cryopreservation: A Review

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Abstract

Advances in sperm assessment techniques have offered new perspectives to improve the technology of semen cryopreservation. This review addresses some recent achievements in the proteomics of seminal plasma and spermatozoa and exemplifies its importance as markers for sperm fertility following cryopreservation. Recent advances in transcriptome studies on sperm RNA-Seq data have generated new information aimed to unravel the physiological roles of RNAs in the sperm-egg fertilization processes and their associations with male fertility. The relevance of the sperm freezability markers and the potential associations of RNA-profiling sequences with the sperm biological functions have been discussed.

Keywords: spermatozoa, frozen-thawed semen, RNA-Seq, bioinformatics studies

1. Introduction

Cryopreservation of semen allows the preservation of good genetic resources and the protection of endangered species [1–5]. While a great deal of efforts has been done over the last several years to improve the semen cryopreservation technology, effective cryosurvival of spermatozoa from various animal species, including the boar and stallion, still remains elusive and a cryobiological enigma [1]. Cryo-induced oxidative stress is associated with excess production of reactive oxygen species (ROS), resulting in biochemical and physical damage to the sperm membrane structures and subsequently leading to reduced fertilizing ability of spermatozoa [4–6]. In the artificial insemination (AI) industry, there is a need to optimize the

selection strategy for individuals with good freezability, so as to incorporate this information in the breeding program to improve the fertility of post-thaw semen [2, 3, 7]. Moreover, selection of animals with good semen freezability for cryopreservation and AI is a crucial step to improve the fertility levels of frozen-thawed semen [9, 10]. Furthermore, in some animal species, despite satisfactory results of fertility in liquid-stored semen, frozen-thawed semen does not give acceptable fertility results in AI practice in the commercial industry [2, 3, 9].

Accumulating evidence has indicated that inherent male variability in semen freezability is one of the factors responsible for marked differences in the sperm cryosurvival [2, 5, 7–10]. With regard to boar semen, studies have reported that differences in sperm freezability might be due to a genetic origin [7, 8]. Even though the underlying mechanisms responsible for the genetic differences associated with poor or good semen freezability are yet unknown, it has been suggested that the identification of sperm freezability markers might be the most efficient approach to improve the technology of semen cryopreservation.

Recent technological advances have confirmed that the spermatozoon carries epigenetic factors that constitute their epigenome, such as proper packaging of the chromatin with protamines, modifications of histones, and a large population of messenger ribonucleic acid (mRNA) and microRNA (miRNA) transcripts [11–13]. The diversity of the RNA constellation in the seminal plasma (SP) and spermatozoa has been used as a pattern for the genomic analysis of semen quality characteristics, particularly for the estimation of the fertility potential of spermatozoa [12–17]. Moreover, high-throughput sequencing demonstrates that several stable full-length mRNA transcripts are useful markers for sperm functions in fresh and frozen-thawed semen [17]. It has been hypothesized that transcriptome analysis of sperm RNA-Sequencing (RNA-Seq) data is required to explore the potential links between semen freezability and the transcript profiles of spermatozoa [18–20]. This review discusses recent accomplishments in molecular markers for the assessment of post-thaw sperm quality and exemplifies the significant relevance of transcriptome profiling by RNA-Seq in semen cryopreservation.

2. Markers for sperm functions

2.1. Motility and motion characteristics

Subjective motility evaluation is one of the most commonly used parameters to determine the quality of frozen-thawed semen for AI. Even though post-thaw sperm motility is a good indicator of viability, it is not always an accurate fertility predictor of an AI-semen dose [9]. Evaluations of sperm motility characteristics have been improved by the incorporation of the computer-assisted semen analysis (CASA) system, which measures several motility and motion parameters of spermatozoa that are closely related to fertility compared with subjective motility measurements [21–24]. Besides motility analysis, studies have confirmed that the velocity parameters, such as velocity straight line (VSL), velocity curvilinear (VCL), and velocity average path (VAP), are associated with the fertilizing capacity of frozen-thawed spermatozoa [21, 24].

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2.2. Membrane integrity

Spermatozoa comprise several compartments enclosed within the acrosome, plasma membrane, and mitochondrial membranes, which act as physiological barriers that must remain intact to permit cell viability, particularly after cryopreservation [1, 2, 6, 10]. In recent years, several fluorescent probes have shown that the cryopreservation process compromises the sperm plasma membrane integrity (PMI), resulting in reduced fertilizing capacity of post-thaw semen [3–5, 23–29]. Post-thaw sperm PMI has been assessed with different fluorescent membrane probes, such as the dual SYBR-14 and propidium iodide (PI) assay [25, 28] or membrane-permeable substrate carboxyfluorescein diacetate (CFDA), a nonspecific esterase substrate [24]. The chlortetracycline (CTC) fluorescence assay has been used to detect capacitation-like changes in frozen-thawed spermatozoa, which may compromise their fertilizing ability [6, 10, 22–24, 29]. Cryo-induced changes in the acrosome membrane integrity (AMI) have been monitored by specific Giemsa-staining technique [25] or with fluorescent dyes, such as fluorescein isothiocyanate (FITC)-conjugated PNA (peanut agglutinin) or conjugated PSA (*Pisum sativum* agglutinin), known as plant lectins, which bind to glycoproteins in the outer acrosomal membrane [4, 5, 26, 27]. Studies have shown that the utilization of a triple staining—SYBR-14, phycoerythrin-conjugated PNA (PE-PNA), and PI (SYBR-14/PE-PNA/PI)—to simultaneously evaluate the PMI and AMI of frozen-thawed bull spermatozoa can be used effectively to assess post-thaw semen viability [26–28]. In a recent study, it has been demonstrated that FITC-PSA/PI-staining protocol can detect marked deterioration in both the PMI and AMI in frozen-thawed bull spermatozoa [4]. Earlier changes in the membrane permeability of frozen-thawed spermatozoa have been monitored with the calcium-dependent binding of Annexin-V/FITC/PI [21, 24, 26] or YO-PRO-1 assay [23, 25, 28], which is an impermeable membrane probe. Moreover, triple staining with YO-PRO-1, ethidium homodimer (Eth), and SNARF-1 (YO-PRO-1/Eth/SNARF-1) has been shown to give similar results with respect to PMI assessment of frozen-thawed spermatozoa compared with the Annexin-V/FITC/PI assay [27, 28]. Kumar et al. [21] reported that there were marked differences in the percentages of frozen-thawed spermatozoa with apoptotic-like changes between fertile and sub-fertile bulls, being significantly higher in the latter. Moreover, there is accumulating evidence indicating that the cryopreservation procedure induces apoptotic-like features in bovine spermatozoa, which appeared as ordered events during the freezing-thawing process, such as reduced mitochondrial membrane potential (MMP), increased caspase activation, and modifications in membrane permeability [6, 21, 24, 27, 28, 30]. Additionally, post-thaw PMI has been assessed with the hypo-osmotic swelling (HOS) test, which evaluates the functional membrane integrity of the acrosome and tail regions when spermatozoa are exposed to hypo-osmotic conditions [22, 24, 29].

Cryopreservation affects the lipid composition and organization of the sperm plasma membranes, resulting in leakage of valuable intracellular enzymes, such as antioxidants [4], acrosin [31], aspartate aminotransferase (AspAT) [32], or energy substrates, such as adenosine triphosphate (ATP) [33], which ultimately lead to cell death. Another measure of membrane damage to frozen-thawed spermatozoa is the degree of lipid peroxidation (LPO) of polyunsaturated fatty acids in sperm cell membranes, induced by the production of reactive oxygen

species during cryopreservation. It has been confirmed that frozen-thawed boar spermatozoa are susceptible to $FeSO_4$/ascorbate-induced LPO, measured by the production of malondialdehyde (MDA), which is capable of triggering apoptotic-like changes that could result in the sublethal sperm cryodamage [24, 30, 32]. Furthermore, the extent of LPO-induced damage to frozen-thawed spermatozoa can be analyzed by monitoring the colorimetric measurements of lipid peroxide formation with a fluorescent membrane probe, $BODIPY^{581/591}$-C11 [26, 30].

The sperm mitochondrial membrane potential is necessary for ATP production, which is the main energy support for several functions [25, 32]. Several studies showed that cryo-induced damage to the MMI of spermatozoa is one the major causes of their reduced fertilizing capacity [4, 5, 10, 24, 25, 29, 33]. These studies detected a marked deterioration in the sperm mitochondrial function following cryopreservation, as reported by the fluorescent staining with rhodamine 123 (R123), the lipophilic cationic compound 5,5′,6,6′-tetrachloro-1,1′,3,3′ tetraethylbenzymidazolyl carbocyanine iodine (JC-1), or with ATP measurements by the bioluminescence assay. The percentages of frozen-thawed spermatozoa with functional mitochondria, as assessed by either the R123/PI or JC-1/PI assay, are highly correlated to motility [5, 29, 33]. Besides the R123/PI and JC-1/PI assays, there are a plethora of fluorescent dyes that can be used to microscopically or cytometrically assess the sperm mitochondrial membrane function (MMF). Some fluorescent MitoTracker probes, such as MitoTracker Deep Red, MitoTracker Red, MitoTracker Orange, and MitoTracker Green, have been used effectively to assess the MMF on frozen-thawed spermatozoa [23, 26]. Boars with good and poor semen freezability ejaculates were identified using several sperm function parameters, including total and progressive motility (TMOT and PMOT, respectively), and rapid movement (RAP) analyzed by the computer-assisted semen analysis system, mitochondrial membrane function, MMF (JC-1/PI assay) and PMI (SYBR-14/PI assay) (**Figure 1**). Post-thaw analysis of the sperm parameters showed that boars with good semen freezability were characterized by significantly higher sperm cryosurvival (Boars 1–6) compared with those with poor semen freezability (Boars 7–10; **Figure 1**), suggesting the importance of these sperm parameters in the assessment of post-thaw semen quality (unpublished results).

2.3. Chromatin and DNA integrity

Sperm chromatin and DNA integrity is an uncompensable trait because abnormalities in the male genome, characterized by damaged chromatin/DNA structure, may be manifested until the sperm-oocyte fusion, or at early embryo development [34]. Accumulating evidence has shown that sperm DNA integrity is one of the parameters of semen quality assessment that has paramount importance in the prognosis of fertility and the outcome of assisted reproductive procedures [5, 34, 35,]. Among the most frequently used sperm DNA integrity assays are the Comet assay (SCGE), which quantifies double-stranded DNA (dsDNA) and single-stranded DNA (ssDNA) breaks under neutral or alkaline electrophoresis [8, 32, 35], the sperm chromatin structure assay (SCSA) measures the susceptibility of sperm chromatin to acid-induced denaturation in situ [34], and the terminal deoxynucleotidyl transferase-mediated dUDP nick end-labeling assay (TUNEL), which quantifies the incorporation of deoxyuridine triphosphate (dUTP) at ssDNA and dsDNA breaks [24, 34]. These DNA integrity assays have confirmed that the cryopreservation process increases the sperm susceptibility to DNA damage, irrespective of the extender or the protocol type [4, 5, 8, 21, 24, 32, 34, 35]. It is worth

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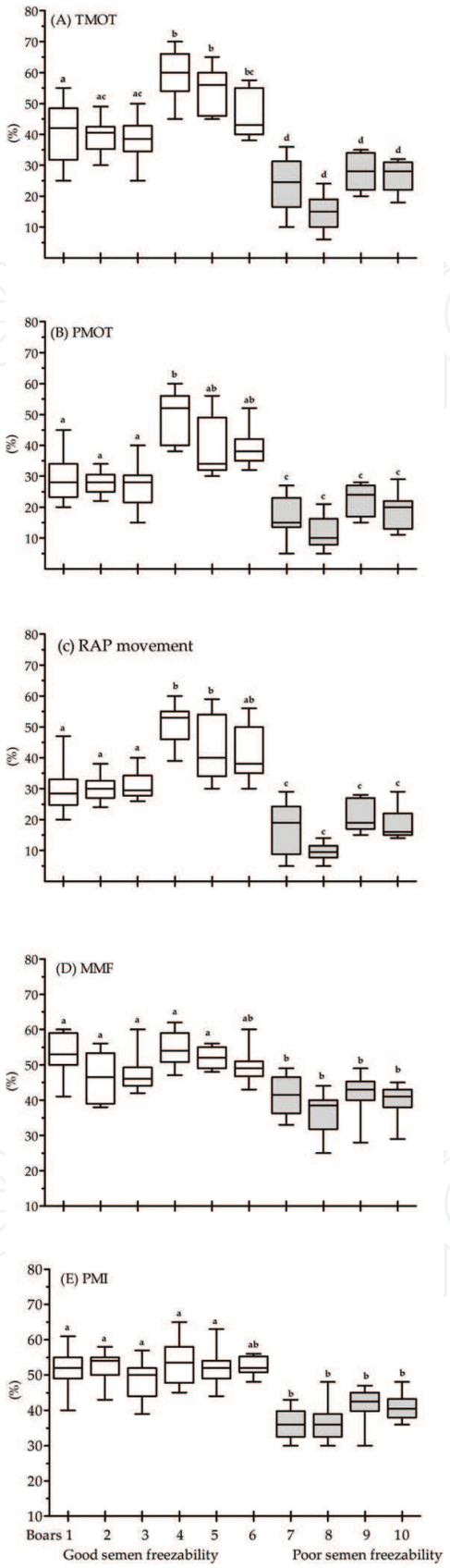

Figure 1. Distribution of the characteristics of frozen-thawed boar spermatozoa for: (A) Total sperm motility (TMOT). (B) Progressive sperm motility (PMOT). (C) Rapid moving (RAP) spermatozoa. (D) Mitochondrial membrane function (MMF). (E) Plasma membrane integrity (PMI) (*unpublished results*). The horizontal lines (–) indicate the medians with 25th and 75th percentile (boxes) and minimum-maximum values (*I*). Values with different letters (a–d) indicate a significant difference ($P < 0.05$).

noting that sperm DNA fragmentation is associated with differential expression of proteins in the viable sperm populations [36]. Intasqui et al. [36] postulated that the overexpression of the sperm proteins in the viable sperm population from ejaculates with high-sperm DNA fragmentation might indicate proteome alterations to compensate defects in sperm motility.

2.4. Protein freezability markers

Recently, seminal plasma and sperm proteins became an integral part of the reproductive area development. It is worth noting that proteins that are involved in the energy metabolism of spermatozoa play key roles in glycolysis, the citric acid cycle, and oxidative phosphorylation, which are required to provide sufficient energy for the sperm physiological functions [37]. The protein constellation of the SP and sperm cells, and their distinct subcompartments have been well documented in a numerous animal species, using a plethora of proteomic-based techniques [6, 37, 38]. While some specific sperm protein markers facilitating good semen freezability have been identified [10, 24, 39, 40], their function depends on the presence of mRNA that can be translated into proteins in the spermatozoa. Moreover, the differential expression patterns of certain classes of SP and sperm proteins following cryopreservation have been used as markers for semen freezability [3, 6, 10, 39–44]. Regarding boar semen, the physiological functions of SP and sperm proteins and their associations with freezability have been summarized in recent reviews by Yeste [3, 10]. In the bull, higher concentrations of a 26-kDa SP protein, known as lipocalin-type prostaglandin D synthase (L-PGDS), and a 13-kDa acidic seminal fluid protein (aSFP) were associated with high-fertility bulls, suggesting the importance of these proteins as freezability markers [41]. Moreover, a fertility-associated protein, osteopontin (OPN), an acidic glycoprotein occurring in the bovine SP, has been shown to induce capacitation and improve viability of spermatozoa by inhibiting the apoptotic pathways [39, 41]. Bovine SP proteins, collectively known as binder of sperm (BSP) proteins (BSP1, BSP 3, and BSP 5), bind to choline phospholipids in the sperm plasma membrane and are implicated in semen freezability [22, 39, 41, 42]. Recently, it has been confirmed that BSP1, one of the most abundantly expressed BSP proteins (representing approximately 25–47% of the total proteins in bovine SP), consists of four molecular forms that have varying cryoprotective effects on the bull spermatozoa [42]. According to Sarsaifi et al. [22], approximately 52% of the SP protein spots detected after cryopreservation were represented by four major protein fractions with different molecular weights, and 10 proteins, identified by mass spectrometry, were major bovine SP proteins. It is worth noting that two of these proteins, defined as phosphoglycerate kinase (PGK, 37–45 kDa) and phospholipase A2 (PLA2, 50–55 kDa), are implicated in glycolysis and the fertilization-associated processes, respectively [22]. More recently, it has been reported that the presence of fertility-associated 28–30-kDa heparin-binding proteins (HPBs) in bovine SP exerted better cryoprotective effects on the sperm structural and functional membrane integrity, which resulted in 13% higher conception rate than the bulls lacking the proteins in their SP [24]. Ledesma et al. [23] postulated that the decrease in phosphotyrosine signal of 45-, 40-, or 30-kDa protein in the presence of SP was concurrent with an inhibition of cryo-induced capacitation of ram spermatozoa, suggesting the relevance of these proteins as markers for the capacitation status of frozen-thawed spermatozoa.

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Spermatozoa have a repertoire of distinct proteins localized in different subcellular structures that are associated with post-thaw semen quality [3, 6, 10, 40, 43]. In boar sperm extracts, the levels of outer dense fiber 2 (ODF2), A-kinase-anchoring protein 3 or 4 (AKAP3; AKAP4), heat-shock protein 90 (HSP90AA1), voltage-dependent anion channel 2 (VDAC2), acrosin-binding protein (ACRBP), and triosephosphate isomerase 1 (TP1) activities were associated with semen freezability [10, 40]. Furthermore, ODFs provide a stable and elastic structure to the flagellum of the spermatozoon, supporting its movement and protecting it during the epididymal transit and ejaculation [36, 39, 40]. The ODF2 seems to be essential to ODF assembly, and its overexpression in frozen-thawed boar spermatozoa was associated with reduced post-thaw semen quality [40]. Moreover, AKAP4 and AKAP3 occur in the fibrous sheath of sperm flagellum and are involved in sperm motility and morphology [36]. It has been confirmed that an increase in the expression of either AKAP4 or AKAP3 in frozen-thawed spermatozoa might be associated with their premature capacitation [40]. In another study, it has been demonstrated that greater levels of HSP90AA1 and VDAC2 in high-freezability boars might confer increased sperm cryotolerance [10]. Furthermore, greater expression levels of a fertility-associated protein, 90-kDa HSP (HSP90), were detected in bull spermatozoa with high cryotolerance and motility, indicating that the protein can be used as a marker for semen freezability [44]. The concept that HSPs supplementation to the freezing extender could protect spermatozoa against cryo-induced damage is supported by a report indicating that the HSPA8, a highly conserved member of the HSP70 family, exerted beneficial effects on post-thaw bull semen quality, as reflected by the reduced proportions of spermatozoa with apoptotic-like changes [45]. According to Chen et al. [40], higher levels of mRNA expression of the membrane proteins cytosolic SOD (Cu/Zn SOD1) in frozen-thawed boar semen might be due to the protective response of the sperm cells to cold stimulation and oxidation stress to prevent cryo-induced damage and also probably due to the toxicity of the components of the cryoprotectants. Recently, the application of high-throughput proteomics to the study of cryopreserved human semen showed substantial changes in the sperm proteome at every stage of the freezing-thawing processes [43]. Irrespective of the thawing procedure of frozen semen, it was reported that there was an increase in the expression levels of a few proteins, such as clusterin (CLU), histone H4 (HIST1H4A), and L-xylulose reductase (DCXR), whereas there was a decrease in the expression levels of several proteins, including apoptosis-inducing factor 1-mitochondrial (AIFM1), carbonic anhydrase 2 (CA2), acrosin (ACR), phosphoglycerate mutase 2 (PGAM2), inositol monophosphatase 1 (IMPA1), calmodulin (CALM1), cytochrome (CYC2), and NADH-cytochrome b5 reductase2 (CYB5R2) [43]. Besides the effect upon the sperm membrane protein P25b, an acrosome membrane-coating protein, cryopreservation causes a significant loss of several sperm-coating proteins, resulting in reduced post-thaw semen quality [10, 22–24, 39–45]. It appears that the cryo-induced decrease in the levels of sperm proteins is probably attributed to protein degradation, membrane damage due to osmotic stress, and the subsequent freezing-thawing causing the efflux of intracellular sperm constituents [6, 43]. Presently, the mechanism responsible for the cryo-induced increase in levels of protein expression is not fully understood, even though it has been suggested that enhanced phosphorylation might be a possible cause for abundance in some of the proteins following cryopreservation [23, 43]. Even though changes in the SP or sperm proteome could predict the cryotolerance of spermatozoa, they do not provide relevant information about possible associations of sperm transcript profiling with semen freezability.

3. Transcriptome studies on sperm-derived RNAs

The records of transcription of the late stages of sperm differentiation are easily accessible through sperm transcript fragments, which have the potential to be used as markers for fertility [13]. Among others, increasing focus has been given to the examination of the biological functions of mRNAs in spermatozoa of different animal species. It has been suggested that the analysis of the sperm-derived RNAs might provide potential links between the sperm proteome and semen freezability [17, 18, 20, 46–48]. It should be underlined that the isolation of high-quality RNAs from fresh or frozen-thawed semen is important to assess the sperm gene expression [19, 46, 49, 50]. However, to optimize the isolation protocol of total RNA from spermatozoa of different animal species, the procedure has to be modified, and should incorporate a quality control reverse transcription polymerase chain reaction (RT-PCR) to avoid somatic cell contamination [17, 19, 46, 49]. Presently, human sperm transcripts are the best characterized among all mammals with respect to RNA sequence profiling. Even though microarray techniques, coupled with either quantitative real-time qPCR or qRT-PCR, have revealed limited features of the transcriptome and global patterns of gene expression in spermatozoa, these techniques have revealed that sperm transcripts affecting different metabolic pathways are related to semen fertility [48, 49, 51–54]. A study based on the evaluation of sperm capacitation status provides evidence, indicating that the sperm-derived RNAs can be translated *de novo* using mitochondrial-type ribosomes, and at least 26 such sperm-translated proteins are known to be required during capacitation, sperm-egg interactions, and fertilization [11, 54].

Recently, the utilization of advanced molecular genetics tools has led to a rapid development of high-throughput RNA-Seq techniques, which have been used to explore the relationship between sperm functions with the transcript profiles of raw fresh or frozen-thawed spermatozoa [12, 50]. The resolution of RNA population has been optimized with the utilization of next-generation sequencing (NGS) technology, to uncover complete transcript profiles of mammalian spermatozoa [12], making significant contributions to elucidate the physiological roles of sperm-derived RNAs. Moreover, the wider adaption of RNA-Seq has revealed a complex RNA repertoire in spermatozoa from different animal species, including the bull [17, 51–53, 55, 56], boar [46–48], and stallion [54]. However, despite the increasingly wide applications of RNA-Seq in the analysis of different cellular tissues, its application is limited to screening of the mRNA profiles of frozen-thawed spermatozoa to uncover candidate genes associated with semen freezability.

Despite its apparent transcriptionally inert state, a mature spermatozoon contains diverse populations of both small and large RNAs [11–13, 47, 55]. Since their discovery in 1989, sperm-derived RNAs were implicated in spermatogenesis and in fertilization and early embryonic development, suggesting that they are not merely the remnants of sperm cell development [11, 46, 54]. Notwithstanding the rich repertoire of coding and noncoding RNAs in mammalian spermatozoa, they are not a random remnant from spermatogenesis in testes, but a selectively retained and functionally coherent collection of RNAs [12, 54, 55]. Spermatozoa contain complex populations of RNAs, including several stable full-length RNAs and a variety of different RNAs, such as ribosome RNAs (rRNA), mitochondrial RNAs (mtRNAs), miRNAs (18–24 nucleotides), piwi-interacting RNAs (piRNAs, 26–31 nucleotides), and small

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interfering RNAs (siRNAs), which originate from double-stranded RNAs (dsRNAs) [11–17, 55]. The precise population of mRNAs in spermatozoa is unknown, but has been estimated to be about 3000–7000 transcripts [17, 54]. It has been established that a majority of sperm mRNAs are located in the nucleus, and a limited number of mRNAs are located in other areas, such as the mid-piece region and flagella fibrous sheath [11]. Individual identified sperm transcripts include mRNAs for ribosomal and mitochondrial proteins, protamines, and proteins involved in signal transduction and cell proliferation [13].

Screening of differentially expressed genes (DEGs) in spermatozoa has been explored mainly to investigate the associations of the gene expression levels with male fertility [11, 14]. Using microarray-based techniques, significant differences in the expression of two genes—testis-specific protein1 (*TPX-1*) and lactate dehydrogenase C, transcript variant (*LDHC*)—were detected in human spermatozoa with high and low motilities [11]. Accumulating evidence has been shown that the coding and noncoding RNAs in spermatozoa play an important role in chromatin stabilization, facilitating the selective escape of sequences necessary for early development from re-packaging by protamines [12, 52, 55]. In human spermatozoa, some putatively sperm transcripts associated with male fertility and early embryo development include *CLU, AKAP4*, Protamine 1 (*PRM1)*, Protamine 2 (*PRM2)*, and heat-shock-binding protein1 (*HSBP1*) [11]. It has been reported that *CLU* binds to the plasma membrane and is the main protein that has been overexpressed in individuals from the low-sperm DNA fragmentation group [36]. Even though the potential role of *CLU* in sperm function is still obscure, it has the potential to be a fertility biomarker for bull, stallion, or human semen [36]. Besides the crucial roles of DEGs in sperm function, there is limited information about the effects of the cryopreservation process on their expression levels. Several full-length transcripts have been identified in the frozen-thawed bull spermatozoa using RNA-Seq [17, 55]. In frozen-thawed bull spermatozoa, highly abundant full-length transcripts, such as *PRM1*, phospholipase C zeta 1 (*PLCZ*1), cysteine-rich secretory protein 2 (*CRISP2*), and calmegin 1 (*CLGN1*), which have been involved in capacitation and fertilization, had been identified using the RNA-Seq [17]. A previous study showed that the transcripts encoding for a serine/threonine testis-specific protein kinase (*TSSK6*) and a metalloproteinase non-coding RNA (*ADAM5P*) were associated with high-motility status in the bull [51].

Significant differences were detected in the DEGs between fresh and frozen-thawed bull spermatozoa using microarray technique in conjunction with qRT-PCR analysis and that upregulations of several DEGs existed, such as ribosomal protein L31 (*RPL31*) and glutamate-cysteine ligase catalytic subunit (*GCLC*), probably due to the protective response of spermatozoa to cold shock and oxidation stress conditions [56]. More recently, an abundance of DEGs has been shown to be highly expressed in bull spermatozoa with poor post-thaw motility compared with those with good post-thaw motility [53]. According to Yathish et al. [53], some of the significantly upregulated DEGs in poor motility freezability ejaculates included the cytochrome b5 reductase 4 (C*YB5R4*), which regulates stress-induced ROS production in spermatozoa; the chaperonin containing T-complex polypeptide 1, subunit 5 (*CCT5*) that is involved in proper folding of cytoskeletal proteins and its high expression is associated with spermatogenesis dysfunction; and *PACSIN3* (protein kinase C and casein kinase substrate in neurons), which is involved in the maintenance of the physiological function of membrane proteins. In a recent

study, the RNA-Seq has detected differences in mRNA transcripts of frozen-thawed semen between high-fertility and low-fertility bulls, and several of these transcripts were considered unique to either fertility group, which differed in their biological functions, such as enrichment of regulatory transcripts for growth and protein kinase activity in the high-fertility bulls [55]. Card et al. [55] demonstrated that the sperm transcript cytochrome oxidase subunit 7C (*COX7C*), which is involved in the terminal step in the electron transport chain leading to ATP synthesis in the inner mitochondrial membrane, was negatively correlated with bull fertility. The authors hypothesized that the abundant expression of COX7C in frozen-thawed spermatozoa from the low-fertility bulls might represent inefficient translation of the transcript, resulting in impaired mitochondrial function during the later stages of spermatogenesis. Similarly, inefficient translation of protein synthesis has been suggested as a possible cause for other sperm transcripts that were abundantly expressed in the low-fertility bulls [55]. However, it should be emphasized that both *PRM1* and *PRM2* are among the most strongly associated transcripts with different semen quality parameters, such as sperm concentration, motility, morphology, and chromatin and DNA integrity, as well as with fertility and embryo quality [11, 17, 46, 52, 55]. Significant reduction in the expression levels of *PRM1* mRNA transcript in post-thaw bull semen was concurrent with compromised progressive sperm motility [52]. It appears that dysfunction in protein synthesis might be associated with aberrant mRNA retention, indicating that the regulation of protamine translation might compromise fertility [11, 52]. Further study, coupling qRT-PCR analysis with an enzyme-linked immunosorbent assay (ELISA), showed that the cryopreservation process caused significant changes in the expression levels of several sperm-derived mRNAs and proteins of epigenetic-related genes in boar spermatozoa [48]. Evidence has been shown that the mRNA expression levels of *PRM1* and *PRM2* were significantly decreased in frozen-thawed bull spermatozoa [52], thus reinforcing the findings of different studies, and indicating that the cryopreservation process induces sperm DNA damage [4, 5, 21, 24, 32, 34, 35]. According to Zeng et al. [48], the mRNA expression levels of *Dnmt3a* and *Dnmt3b*, which are DNA methyl transferases known to possess *de novo* methylation activity in mammalian cells, were markedly suppressed in frozen-thawed spermatozoa, reaffirming the unfavorable effects of the cryopreservation process on post-thaw sperm survival. Furthermore, different transcripts of HSPs have been detected in spermatozoa of various animal species, particularly the most abundantly expressed 70-kDa HSP, which is an effective marker for sperm cryotolerance and might be associated with post-thaw semen quality [45, 57]. Furthermore, seasonality is an important factor that affects the mRNA profiles of boar spermatozoa, resulting in marked differences in the expression levels of genes, which are implicated in numerous sperm physiological and biochemical functions [47]. These findings corroborate those of a recent study, indicating that the marked differences in the gene expression profiles of SP and sperm proteome, in response to seasonal changes, significantly affected the biochemical composition of boar semen, which could compromise post-thaw semen quality [58].

Currently, the biological roles of small-nuclear RNAs (snRNAs)—miRNAs, piRNAs, and siRNAs—which are expressed specifically and abundantly in spermatogenic cells, have been documented by several authors [11–17]. Quantitative RT-PCR analysis on boar sperm RNA revealed that the mRNA targets of the differentially expressed miRNAs encode proteins previously described to play specific roles in sperm function, such as motility and capacitation [14]. According to Chang et al. [16], the differential expression of 15 miRNAs between the cauda

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epididymal spermatozoa and fresh ejaculate in the boar suggests that significant mRNA expression and miRNA regulation are implicated in apoptosis, and are associated with the sperm maturation processes. The authors postulated that the targeted gene, adrenoceptor beta 2 (*ADRB2*), a member of the G-protein-coupled receptor superfamily, activates adenylyl cyclase leading to the activation of cAMP-dependent kinase, whereas another targeted gene, the adenylate cyclase 3 (*ADCY3*), catalyzes the formation of the second messenger cAMP, which in turn leads to elevated cAMP levels. It should be emphasized that while many miRNAs are conserved among different animal species, some of them are species-specific [12]. Furthermore, profiling of the SP by Illumina high-throughput sequencing showed that piRNA appears to play more important and direct roles in spermatogenesis and male infertility, and its expression was significantly reduced in the SP of infertile patients compared with the healthy individuals [12, 13, 15]. However, it remains unclear how individual miRNAs and siRNAs in spermatozoa function at the molecular levels, and the impact of the cryopreservation process on their biological functions. It should be emphasized that the SP and mature spermatozoa contain a plethora of other small RNAs in which their roles in sperm function are currently unknown [13].

4. Concluding remarks

Even though proteins of the SP and spermatozoa have been used as semen freezability markers, these expectations are over-shadowed by the problems associated with the inherent male variability in sperm cryosurvival. The search for a new set of freezability markers using transcriptome studies on RNA-Seq data, bioinformatics study, and proteome characterization of protein expression patterns in frozen-thawed spermatozoa will offer new perspectives to enhance the marker-assisted selection programs in animal breeding. It is envisaged that such freezability markers will also help to unravel the biological functions of sperm-derived mRNA transcripts in the mechanism underlying cryotolerance of spermatozoa from various domestic animal species, and will have a significant impact in the technology of semen cryopreservation.

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References

[1] Benson JD, Woods EJ, Walters EM, Crister JK. The cryobiology of spermatozoa. Theriogenology. 2012;**78**(8):1682-1699. DOI: 10.1016/j.theriogenology.2012.06.007

[2] Prien S, Iacovides S. Cryoprotectants & cryopreservation of equine semen: A review of industry cryoprotectants and the effects of cryopreservation on equine semen membranes. Journal of Dairy, Veterinary & Animal Research. 2016;**3**(1):00063, 1-8. DOI: 10.15406/jdvar.2016.03.00063

[3] Yeste M. Recent advances in boar sperm cryopreservation: State of the art and current perspectives. Reproduction in Domestic Animals. 2015;**50**(Suppl 2):71-79. DOI: 10.1111/rda.12569

[4] Castro LS, Hamilton TR, Mendes CM, Nichi M, Barnabe VH, Visintin JA, Assumpção ME. Sperm cryodamage occurs after rapid freezing phase: Flow cytometry approach and antioxidant enzymes activity at different stages of cryopreservation. Journal of Animal Science and Biotechnology. 2016;**5**(7):1-9. DOI: 10.1186/s40104-016-0076-x

[5] Gürler H, Malama E, Heppelmann M, Calisici O, Leiding C, Kastelic JP, Bollwein H. Effects of cryopreservation on sperm viability, synthesis of reactive oxygen species and DNA damage of bovine sperm. Theriogenology. 2016;**86**:562-571. DOI: 10.1016/j.theriogenology.2016.02.007

[6] Leahy T, Gadella BM. Sperm surface changes and physiological consequences induced by sperm handling and storage. Reproduction. 2011;**142**:759-778. DOI: 10.1530/REP-11-0310

[7] Thurston LM, Siggins K, Mileham AJ, Watson PF, Holt WV. Identification of amplified restriction fragment length polymorphism markers linked to genes controlling boar sperm viability following cryopreservation. Biology of Reproduction. 2002;**66**(3):545-554. DOI: 10.1095/biolreprod66.3.545

[8] Fraser L, Pareek CS, Strzeżek J. Identification of amplified fragment length polymorphism (AFLP) markers associated with freezability of boar semen – a preliminary study. Medycyna Weterynaryjna. 2008;**64**:646-649

[9] Roca J, Broekhuijse MLWJ, Parrilla I, Rodríguez-Martínez H, EA Martinez EA, Bolarin A. Boar differences in artificial insemination outcomes: Can they be minimized? Reproduction in Domestic Animals. 2015;**50**(Suppl. 2):48-55. DOI: 10.1111/rda.12530

[10] Yeste M. Sperm cryopreservation update: Cryodamage, markers, and factors affecting the sperm freezability in pigs. Theriogenology. 2016;**85**:47-64. DOI: 10.1016/j.theriogenology.2015.09.047

[11] Dadoune JP. Spermatozoal RNAs. What about their functions? Microscopy Research and Technique. 2009;**72**:536-551. DOI: 10.1002/jemt.20697

[12] Jodar M, Selvaraju S, Sendler E, Diamond MP, Krawetz SA. The presence, role and clinical use of spermatozoal RNAs. Human Reproduction Update. 2013;**19**:604-624. DOI: 10.1093/humupd/dmt031.

[13] Casas E, Vavouri T. Sperm epigenomics: Challenges and opportunities. Frontiers in Genetics. 2014;5:Article 330, 1-7. DOI: 10.3389/fgene.2014.00330

[14] Curry E, Safranski TJ, Pratta SL. Differential expression of porcine sperm microRNAs and their association with sperm morphology and motility. Theriogenology. 2011;**76**:1532-1539. DOI: 10.1016/j.theriogenology.2011.06.025

[15] Hong Y, Wang C, Fu Z, Liang H, Zhang S, Lu M, Sun W, Ye C, Zhang CY, Zen K, Shi L, Zhang C, Chen X. Systematic characterization of seminal plasma piRNAs as molecular biomarkers for male infertility. Scientific Reports. 2016;**6**:24229, 1-10. DOI: 10.1038/srep24229

[16] Chang Y, Dai DH, Li Y, Zhang Y, Zhang M, Zhou GB, Zeng CJ. Differences in the expression of microRNAs and their predicted gene targets between cauda epididymal and ejaculated boar sperm. Theriogenology. 2016;**86**(9):2162-2171. DOI: 10.1016/j.theriogenology.2016.07.012

[17] Card CJ, Anderson EJ, Zamberlan S, Krieger KE, Kaproth M, Sartini BL. Cryopreserved bovine spermatozoal transcript profile as revealed by high-throughput ribonucleic acid sequencing. Biology of Reproduction. 2013;**88**(1):1-9. DOI: 10.1095/biolreprod.112.103788

[18] Fraser L. A novel approach to assess semen freezability. Veterinary Medicine – Open Journal. 2016;**1**(2):e5-e6. DOI: 10.17140/VMOJ-1-e003

[19] Fraser L, Brym P, Pareek CS. Isolation of total RNA from fresh and frozen-thawed boar semen and its relevance in transcriptome studies. South African Journal of Animal Science. 2017;**47**(1):56-60. DOI: 10.4314/sajas.v47i1.5

[20] Fraser L. Sperm transcriptome profiling for assessment of boar semen freezability. IJASRM. 2016;**1**(12):9-12

[21] Kumar D, Kumar P, Singh P, Yadav SP, Sarkar SK, Bharadwaj A, Yadav PS. Characteristics of frozen thawed semen in predicting the fertility of buffalo bulls. Indian Journal of Animal Science. 2014;**84**(4):389-392

[22] Sarsaifi K, Vejayan J, Haron AW, Yusoff R, Hani H, Rasoli M, Omar MA, Othman AM. Protein profile and functionality of spermatozoa from two semen collection methods in Bali bulls. Life Science. 2015;**172**:96-105. DOI: 10.1016/j.livsci.2014.12.004

[23] Ledesma A, Fernández-Alegre E, Cano A, Hozbor F, Martínez-Pastor F, Cesarid A. Seminal plasma proteins interacting with sperm surface revert capacitation indicators in frozen-thawed ram sperm. Animal Reproduction Science. 2016;**173**:35-41. DOI: 10.1016/j.anireprosci.2016.08.007

[24] Karunakaran M, Devanathan TG. Evaluation of bull semen for fertility-associated protein, in vitro characters and fertility. Journal of Applied Animal Research. 2017;**45**(1):136-144. DOI: 10.1080/09712119.2015.1129343

[25] Wasilewska K, Zasiadczyk Ł, Fraser L, Mogielnicka-Brzozowska M, Kordan W. The benefits of cooling boar semen in different long-term extenders prior to cryopreservation on sperm quality characteristics. Reproduction in Domestic Animals. 2016;**51**(5):781-788. DOI: 10.1111/rda.12751

[26] Hossain MdS, Johannisson A, Wallgren M, Nagy S, Siqueira AP, Rodríguez-Martinez H. Flow cytometry for the assessment of animal sperm integrity and functionality: State of the art. Asian Journal of Andrology. 2011;**13**:406-419. DOI: 10.1038/aja.2011.15

[27] Nagy S, Jansen J, Topper EK, Gadella BM. A triple-stain flow cytometric method to assess plasma- and acrosome-membrane integrity of cryopreserved bovine sperm immediately after thawing in presence of egg-yolk particles. Biology of Reproduction. 2003;**68**:1828-1835. DOI: 10.1095/biolreprod.102.011445

[28] Peña FJ. Detecting subtle changes in sperm membranes in veterinary andrology. Asian Journal of Andrology. 2007;**9**:731-737. DOI: 10.1111/j.1745-7262.2007.00311.x

[29] Yoon SJ, Kwon WS, Rahman MdS, Lee JS, Pang GM. A novel approach to identifying physical markers of cryo-damage in bull spermatozoa. PLoS One. 2015;**10**(5):e0126232. DOI: 10.1371/journal.pone.0126232

[30] Ferrusola O, Fernández LG, Morrell JM, Sandoval CS, Garcia BM, Tapia JA, Rodríguez-Martínez H, Tapia JA, Peña FJ. Lipid peroxidation, assessed with BODIPY-C_{11}, increases after cryopreservation of stallion spermatozoa, is stallion-dependent and is related to apoptotic-like changes. Reproduction. 2009;**138**:55-63. DOI: 10.1530/REP-08-0484

[31] Pinart E, Yeste M, Bonet S. Acrosin activity is a good predictor of boar sperm freezability. Theriogenology. 2015;**83**:1525-533. DOI: 10.1016/j.theriogenology.2015.02.005

[32] Fraser L, Zasiadczyk Ł, Pareek CS. Effects of boar variability on comet-detected sperm DNA damage following cryopreservation. Animal Production Science. 2016. DOI: 10.1071/AN16274

[33] Fraser L, Dziekońska A, Strzeżek R, Strzeżek J. Dialysis of boar semen prior to freezing thawing: Its effects on post-thaw sperm characteristics. Theriogenology. 2007;**67**:994-1003. DOI: 10.1016/j.theriogenology.2006.12.002

[34] Evenson DP. The sperm chromatin structure assay (SCSA®) and other sperm DNA fragmentation tests for evaluation of sperm nuclear DNA integrity as related to fertility. Animal Reproduction Science. 2016;**169**:56-75. DOI: 10.1016/j.anireprosci.2016.01.017

[35] Fraser L, Parda A, Filipowicz K, Strzeżek J. Comparison of post-thaw DNA integrity of boar spermatozoa assessed with the neutral Comet assay and sperm-Sus Halomax test kit. Reproduction in Domestic Animals. 2010;**45**:e155-e160. DOI: 10.1111/j.1439-0531.2009.01537.x

[36] Intasqui P, Camargo M, Del Giudice PT, Spaine DM, Carvalho VM, Cardozo KHM, Cedenho AP, Bertolla RP. Unraveling the sperm proteome and post-genomic pathways associated with sperm nuclear DNA fragmentation. Journal of Assisted Reproduction and Genetics. 2013;**30**:1187-1120. DOI: 10.1007/s10815-013-0054-6

[37] Holland A, Ohlendieck K. Comparative profiling of the sperm proteome. Proteomics. 2015;**15**:632-648. DOI: 10.1002/pmic.201400032

[38] Perez-Patiño C, Barranco I, Parrilla I, Valero ML, Martinez EA, Rodriguez-Martinez H, Roca J. Characterization of the porcine seminal plasma proteome comparing ejaculate portions. Journal of Proteomics. 2016;**142**:15-23. DOI: 10.1016/j.jprot.2016.04.026

[39] Moura AA, Memili E. Functional aspects of seminal plasma and sperm proteins and their potential as molecular markers of fertility. Animal Reproduction. 2016;**13**(3):191-199. DOI:10.21451/19843143-AR884

[40] Chen X, Zhu H, Hu C, Hao H, Zhang J, Li K, Zhao X, Qin T, Zhao K, Zhu H, Wang D. Identification of differentially expressed proteins in fresh and frozen–thawed boar spermatozoa by iTRAQ-coupled 2D LC–MS/MS. Reproduction. 2014;**147**:321-330. DOI: 10.1530/REP-130313

[41] Kumar P, Kumar D, Singh I, Yadav PS. Seminal plasma proteome: Promising biomarkers for bull fertility. Agricultural Research. 2012;**1**(1):78-86. DOI: 10.1007/s40003-011-0006-2

[42] Magalhães MJ Jr, Martins LF, Senra RL, Santos TF, Okano DS, Pereira PR, Faria-Campos A, Campos SV, Guimarães JD, Baracat-Pereira MC. Differential abundances of four forms of Binder of SPerm 1 in the seminal plasma of *Bos taurus in*dicus bulls with different patterns of semen freezability. Theriogenology. 2016;**86**(3):766-777. DOI: 10.1016/j.theriogenology.2016.02.030

[43] Bogle OA, Kumar K, Attardo-Parrinello C, Lewis SEM, Estanyol JM, Ballesc JL, Oliva R. Identification of protein changes in human spermatozoa throughout the cryopreservation process. Andrology. 2017;**5**:10-22. DOI: 10.1111/andr.12279

[44] Wang P, Wang YF, Wang H, Wang CW, Zan LS, Hu JH, Li QW, Jia YH, Guo-Ji Ma GJ. HSP90 expression correlation with the freezing resistance of bull sperm. Zygote. 2014;**22**:239-245. DOI: 10.1017/S096719941300004x

[45] Holt WV, Del Valle DI, Fazeli A. Heat shock protein A8 stabilizes the bull sperm plasma membrane during cryopreservation: Effects of breed, protein concentration, and mode of use. Theriogenology. 2015;**84**:693-701. DOI: 10.1016/j.theriogenology.2015.05.004

[46] Yang CC, Lin YS, Hsu CC, Wu SC, Lin EC, Cheng WTK. Identification and sequencing of remnant messenger RNAs found in domestic swine (*Sus scrofa*) fresh ejaculated spermatozoa. Animal Reproduction Science. 2009;**113**:143-155. DOI: 10.1016/j.anireprosci.2008.08.012

[47] Yang CC, Lin YS, Hsu CC, Tsai MH, Wu SC, Cheng WTK. Seasonal effect on sperm messenger RNA profile of domestic swine (*Sus Scrofa*). Animal Reproduction Science. 2010;**119**(1-2):76-84. DOI: 10.1016/j.anireprosci.2009.12.002

[48] Zeng C, Peng W, Ding L, He L, Zhang Y, Fang D, Tang K. A preliminary study on epigenetic changes during boar spermatozoa cryopreservation. Cryobiology. 2014;**69**:119-127. DOI: 10.1016/j.cryobiol.2014.06.003

[49] Han CM, Chen R, Li T, Chen XL, Zheng YF, Ma MT, Gao QH. Evaluation of the semen swim-up method for bovine sperm RNA extraction. Genetics and Molecular Research. 2016;**15**(2):1-9. DOI: 10.4238/gmr.15027713

[50] Schuster A, Tang C, Xie Y, Ortogero N, Yuan S, Yan W. SpermBase – A database for sperm borne RNA contents. Biology of Reproduction. 2016;**95**(5):99, 1-12. DOI: 10.1095/biolreprod.116.142190

[51] Bissonnette N, Levesque-Sergerie JP, Thibault C, Boissonneault G. Spermatozoal transcriptome profiling for bull sperm motility: A potential tool to evaluate semen quality. Reproduction. 2009;**38**:65-80. DOI: 10.1530/REP-08-0503

[52] Ganguly I, Gaur GK, Kumar S, Mandal DK, Kumar M, Singh U, Kumar S, Sharma A. Differential expression of protamine 1 and 2 genes in mature spermatozoa of normal and motility impaired semen producing crossbred Frieswal (HF × Sahiwal) bulls. Research in Veterinary Science. 2013;**94**:256-262. DOI: 10.1016/j.rvsc.2012.09.001

[53] Yathish HM, Kumar S, Dubey PP, Modi RP, Chaudhary R, Kumar AS, Ghosh SK, Sarkar M, Sivamani B. Profiling of sperm gene transcripts in crossbred (*Bos taurus* × *Bos indicus*) bulls. Animal Reproduction Science. 2017;**177**:25-35. DOI: 10.1016/j.anireprosci.2016.12.003

[54] Das PJ, McCarthy F, Vishnoi M, Paria N, Gresham C, Gang L, Kachroo P, Sudderth AK, Teague S, Love CC, Varner DD, Chowdhary BP, Raudsepp T. Stallion sperm transcriptome comprises functionally coherent coding and regulatory RNAs as revealed by microarray analysis and RNA seq. PLoS One. 2013;**8**(2):1-15, e56535. DOI:10.1371/journal.pone.0056535

[55] Card CJ, Kreiger KE, Kaproth M, Sartini BL. Oligo-dT selected spermatozoal transcript profiles differ among higher and lower fertility dairy sires. Animal Reproduction Science. 2017;**177**:105-123. DOI: 10.1016/j.anireprosci.2016.12.011

[56] Chen X, Wang Y, Zhu H[1], Hao H, Zhao X, Qin T, Wang D. Comparative transcript profiling of gene expression of fresh and frozen-thawed bull sperm. Theriogenology. 2015;**4**:504-511. DOI: 10.1016/j.theriogenology.2014.10.015

[57] Zhang XG, Hong JY, Yan GJ, Wang YF, Li QW, Hu JH. Association of *heat shock pro*tein 70 with motility of frozen-thawed sperm in bulls. Czech Journal of Animal Science. 2015;**6**0(6):256-262. DOI: 10.17221/8239-CJAS

[58] Fraser L, Strzeżek J, Filipowicz K, Mogielnicka-Brzozowska M, Zasiadczyk Ł. Age and seasonal-dependent variations in the biochemical composition of boar semen. Theriogenology. 2016;**86**:806-816. DOI: 10.1016/j.theriogenology.2016.02.035